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Paclitaxel Enhances the Effects of the Anti-Epidermal Growth Factor Receptor Monoclonal Antibody ImClone C225 in Mice with Metastatic Human Bladder Transitional Cell Carcinoma¹

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ABSTRACT

Previously we reported that when cells from the human transitional cell carcinoma cell line 253J B-V growing orthotopically within the bladder of athymic nude mice were treated with the anti-epidermal growth factor receptor monoclonal antibody C225, angiogenesis was inhibited, resulting in regression of the primary tumor and inhibition of metastasis. In this study, we evaluated whether paclitaxel enhanced this therapeutic effect of C225. *In vitro*, the proliferation of 253J B-V cells was inhibited more by the combination of C225 and paclitaxel than with either agent alone. *In vivo* therapy with C225 and paclitaxel resulted in significantly greater regression of tumors compared with either agent alone. Median bladder tumor weight was 85 mg (range, 69-133 mg) compared with 168 mg (range, 72-288 mg) after C225 alone ($P < 0.05$), and 273 mg (range, 83-563 mg) after paclitaxel alone ($P < 0.005$). The incidence of spontaneous lymph node metastasis was also reduced by the combination of C225 with paclitaxel, although this result did not significantly differ from results after the use of C225 alone. Treatment with paclitaxel and C225 down-regulated the expression of basic fibroblast growth factor, vascular endothelial cell growth factor, interleukin-8, and matrix metalloproteinase type 9 and inhibited tumor-induced neovascularity compared with untreated controls ($P < 0.005$). Moreover, the combination of C225 and paclitaxel enhanced

apoptosis in tumor and endothelial cells compared with either agent alone ($P < 0.005$). These studies indicate that therapy with paclitaxel increases the ability of C225 to inhibit tumorigenicity and metastasis. This effect is mediated by inhibition of angiogenesis and induction of apoptosis.

INTRODUCTION

TCC³ of the bladder, the fourth most common malignancy in the United States, is diagnosed in ~54,000 persons and results in 12,000 deaths annually (1). Clinical studies evaluating the significance of EGFR expression in human TCC have shown that >50% of human TCCs overexpress EGFR and that the level of expression directly correlates with tumor grade, stage, and survival (2-6). In superficial bladder cancer, EGFR expression correlates with multiplicity, time to disease recurrence, and overall recurrence rates (2-4). EGFR expression also predicts for disease progression from superficial to muscle-invasive or metastatic TCC and is an independent prognostic factor for death from superficial TCC (2, 5, 6). EGFR expression is also an important prognostic factor for patients with advanced TCC, and patients with muscle-invasive TCCs that overexpress EGFR have only a 20% probability of long-term cancer-specific survival (3-6). These results have shown the importance of EGFR expression for the growth, invasion, and subsequent metastasis of TCC.

We recently reported results of a study in which human bladder TCC established orthotopically in nude mice was treated with the anti-EGFR monoclonal antibody C225 (7). In this study, C225 inhibited tumor growth and metastasis by inhibiting tumor-induced neovascularization by down-regulating expression of the angiogenic factors VEGF, IL-8, and bFGF by the tumor cells. Treatment with C225 also down-regulated expression of MMP-9 and induced apoptosis within the regressing tumors.

Previous studies have shown that therapy with C225 or the anti-Her2/nev receptor antibody interceptor was enhanced by combination with cisplatin (8, 9), topotecan (10), doxorubicin (11, 12), or paclitaxel (12). Paclitaxel inhibits cell replication by enhancing polymerization of tubulin monomers into stabilized

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³ The abbreviations used are: TCC, transitional cell carcinoma; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial cell growth factor; IL-8, interleukin-8; bFGF, basic fibroblast growth factor; MMP-9, matrix metalloproteinase type 9; FBS, fetal bovine serum; PI, propidium iodide; ISH, *in situ* hybridization; IHC, immunohistochemical staining; PCNA, proliferating cell nuclear antigen; MVD, microvessel density; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling.

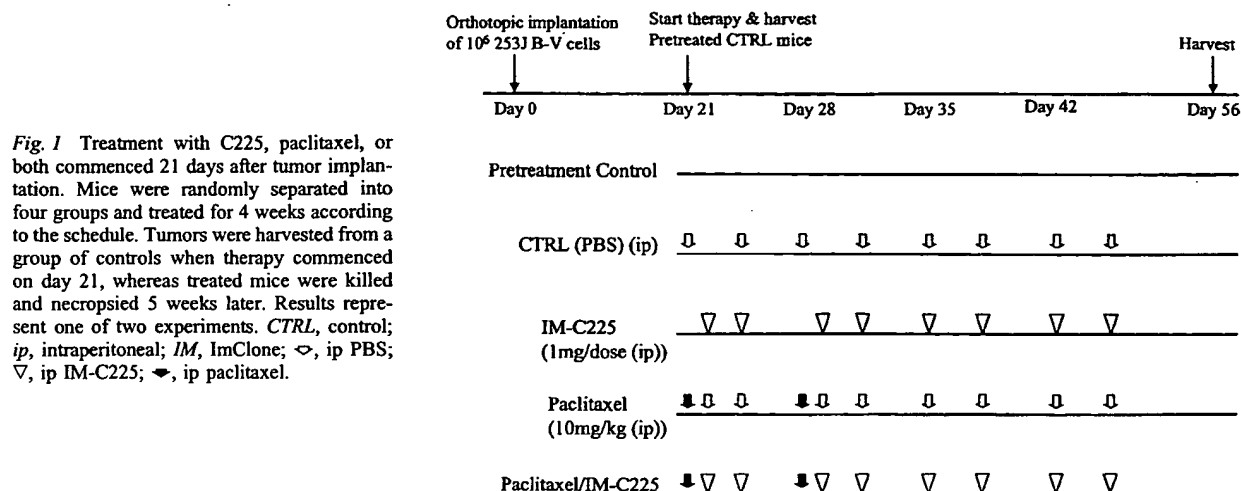


Fig. 1 Treatment with C225, paclitaxel, or both commenced 21 days after tumor implantation. Mice were randomly separated into four groups and treated for 4 weeks according to the schedule. Tumors were harvested from a group of controls when therapy commenced on day 21, whereas treated mice were killed and necropsied 5 weeks later. Results represent one of two experiments. CTRL, control; ip, intraperitoneal; IM, ImClone; \square , ip PBS; ∇ , ip IM-C225; \blacksquare , ip paclitaxel.

microtubule bundles that are unable to reorganize into the proper structures for mitosis (13, 14), resulting in cell cycle blockade in mitosis and subsequent activation of an apoptotic pathway (15, 16). Paclitaxel has also been shown to be an active agent for the treatment of advanced TCC (17, 18). We therefore evaluated the antitumor effect of C225 and paclitaxel used together as therapy for metastatic TCC growing within the bladder of athymic nude mice.

MATERIALS AND METHODS

Cell Culture. The highly metastatic human bladder carcinoma cell line 253J B-V was grown as a monolayer in modified Eagle's MEM supplemented with 10% FBS, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (19).

Reagents. The chimeric (mouse-human) anti-EGFR monoclonal antibody C225 was generously provided by ImClone Systems, Inc. (New York, NY). Paclitaxel was purchased from Bristol-Myers Squibb Co. (Princeton, NJ).

In Vitro Growth Inhibition. We evaluated the *in vitro* dose-dependent antiproliferative effect of C225 and paclitaxel by incubating 1×10^3 253J B-V cells for 24 h in serum-free medium, and then changing the medium to 10% FBS-supplemented MEM containing increasing concentrations of C225 (0–1000 μ g/ml) and/or paclitaxel (0–1000 μ g/ml), or both. Growth inhibition was determined after 72 h by [3 H]thymidine incorporation.

In Vitro Apoptosis. To evaluate the *in vitro* dose-dependent apoptotic effect of C225 and paclitaxel, we incubated 5×10^5 253J B-V cells for 24 h in serum-free medium and then in 10% FBS-supplemented MEM containing increasing concentrations of C225 (0–1000 μ g/ml) and/or paclitaxel (0–1000 μ g/ml), or both. Cells were harvested by centrifugation and stained by incubation at 4°C for 24 h in PBS containing 50 μ g/ml PI. DNA fragmentation was quantified by flow cytometry with PI staining.

Animals. Male athymic BALB/c nude mice were obtained from the Animal Production Area of the National Cancer

Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar air-flow cabinet under pathogen-free conditions and used at 8–12 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the NIH.

Orthotopic Implantation of Tumor Cells. Cultured 253J-BV cells (60–70% confluent) were prepared for injection as described previously (19). Mice were anesthetized with methoxyflurane. A lower midline incision was made, and viable tumor cells (1×10^6 cells in 0.05 ml of HBSS) were orthotopically implanted into the bladder wall on day 0. The formation of a bulla indicated a satisfactory injection. The bladder was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips.

In Vivo Treatment of Established Human TCC Growing in the Bladders of Athymic Nude Mice. Treatment began on day 21. Tumors were harvested from a group of controls ($n = 5$) on day 21 prior to initiation of therapy. Mice were randomly separated into four groups ($n = 10$) and treated for 4 weeks according to the schedule shown in Fig. 1. Mice then were killed and necropsied on day 56. The primary tumors were removed and weighed, and the presence of metastases (in lymph nodes and lung) was determined grossly and microscopically. The bladders were then quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, placed in OCT compound (Miles Laboratories, Elkhart, IN), and frozen at -80°C or mechanically dissociated and put into tissue culture. The lungs and lymph nodes were fixed in 10% buffered formalin or mechanically dissociated and put into tissue culture.

In Situ mRNA Hybridization Analysis. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequence: EGFR (5'-CGA GCG CTG CCC CGG CCG TCC CGG-3'), 87.5% GC content (2); bFGF (5'-CGG GAA GGC GCC GCT GCC GCC-3'), 85.7% GC content (20);

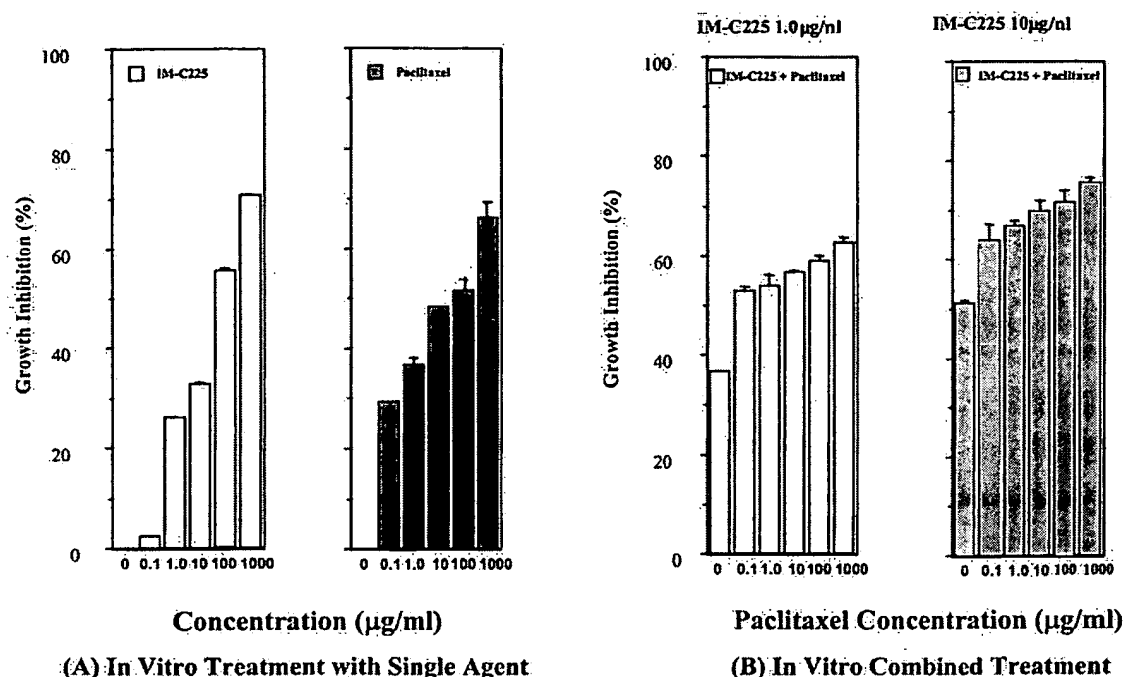


Fig. 2 *In vitro* cell growth inhibition by either C225 or paclitaxel resulted in dose-dependent cytostasis as measured by [3 H]thymidine incorporation assay. The IC_{50} s of C225 and paclitaxel were 100 and 10 µg/ml, respectively (A). Combined treatment with both C225 and paclitaxel inhibited 70% of cell growth at 10 and 100 µg/ml, respectively (B). Results represent one of two experiments; bars, SD. IM, ImClone.

VEGF/vascular permeability factor (5'-TGG TGA TGT TGG ACT CTT CAG TGG GCU-3'), 57.7% GC content (21); IL-8 (5'-CTC CAC AAC CCT CTG CAC CC-3'), 66% GC content (22); MMP-9 (5'-CCG GTC CAC CTC GCT GGC GCT CCG GU-3'), 80.0% GC content (23). The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis (24).

A poly(dT)₂₀ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3' end via direct coupling, with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted to a stock solution at 1 µg/µl in 10 mM Tris (pH 7.6), 1 mM EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics).

In situ mRNA hybridization was performed as described previously with minor modifications (25, 26). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 27). Tissue sections (4 µm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 25, 26). The slides were placed in the Microprobe slide holder, dewaxed,

and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at 45°C, and the samples were then washed three times with 2× SSC for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with a chromogen substrate for 15 min at 45°C. If necessary to enhance a weak reaction, samples were incubated a second time with fresh chromogen substrate. A red stain indicated a positive reaction. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

Quantification of Color Reaction. Stained sections were examined using a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony Corp., Tokyo, Japan). The images were analyzed using Optimas image analysis software (version 4.10; Media Cybernetics, Silver Spring, MD). The slides were prescreened by one of the investigators to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on its equivalence to the mean log-inverse gray value multiplied by the area of the field. The samples were not counterstained, so the absorbance was attrib-

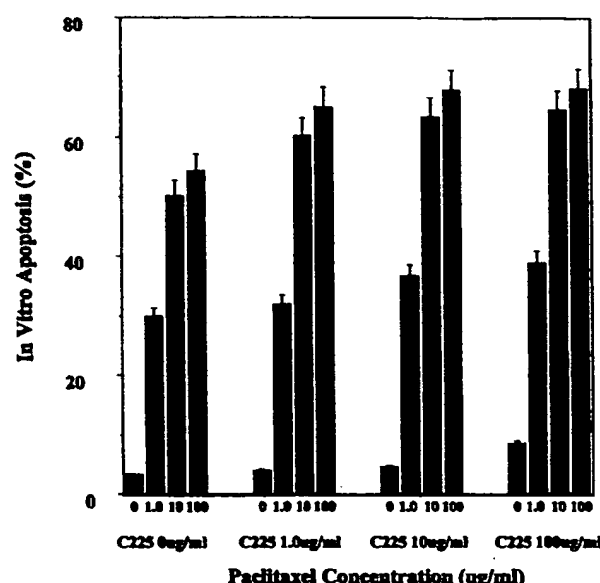


Fig. 3 The dose-dependent apoptotic effect of C225 and/or paclitaxel on 253J B-V cells was determined by flow cytometry using PI. The results are expressed as the percentage of apoptotic cells per all incubated cells. Treatment with paclitaxel induced apoptosis in 29.9, 50.3, and 54.4% of cells at concentrations of 1, 10, and 100 $\mu\text{g/ml}$, respectively, whereas treatment with C225 caused apoptosis in only 3.9, 4.6, and 8.5% of cells at concentrations of 1, 10, and 100 $\mu\text{g/ml}$. A combination of drugs at their IC_{50} doses (100 $\mu\text{g/ml}$ for C225 and 10 $\mu\text{g/ml}$ for paclitaxel) had an additive effect, with apoptosis induced in 64.5% of cells. Results represent one of two experiments; bars, SD.

utable solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated absorbance of poly(dT)₂₀. The results were presented as the intensity of expression for each treatment group compared with the control, which was set to 100 (7).

IHC. For IHC analysis, frozen tissue sections (8 μm thick) were fixed with cold acetone. Tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS, antigen retrieval was performed with pepsin for 12 min, and endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution containing PBS (pH 7.5), 5% normal horse serum, and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with one of the following: a 1:100 dilution of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; Ref. 28), a 1:500 dilution of rabbit polyclonal anti-bFGF antibody (Sigma Chemical Co., St. Louis, MO), a 1:500 dilution of rabbit polyclonal anti-VEGF/vascular permeability factor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:50 dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products,

Table 1 Combination chemotherapy of anti-EGFR monoclonal antibody C225 and/or paclitaxel for human TCC 253J B-V growing orthotopically in athymic nude mice

Mice received implants containing 1×10^6 cells, and treatment commenced 21 days later. Five mice were sacrificed at the initiation of therapy, and the remaining mice were randomly separated into four groups (shown in Fig. 1). All mice were sacrificed 5 weeks after initiation of therapy.

Therapy	Incidence	Tumorigenicity median bladder weight (range), mg	Lymph node metastasis incidence
Pretreatment CTRL ^a	4/4	269 (144–378)	1/4
CTRL (PBS)	9/9	481 (377–877)	9/9
Paclitaxel	9/9	273 (83–563) ^b	7/9
C225	7/7	168 (72–288) ^c	2/7 ^c
Paclitaxel/C225	7/7	85 (69–133) ^d	0/7 ^c

^a CTRL, control.

^{b-d} Mann-Whitney statistical comparison and χ^2 test: ^b $P < 0.005$ vs. CTRL; ^c $P < 0.0005$ vs. CTRL and < 0.05 vs. paclitaxel; ^d $P < 0.0005$ vs. CTRL, < 0.005 vs. paclitaxel, and < 0.05 vs. C225.

Cambridge, MA), or a 1:100 dilution of mouse monoclonal anti-PCNA antibody (DAKO, Carpinteria, CA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody: peroxidase-conjugated antirat IgG (H+L; Jackson ImmunoResearch Laboratory, West Grove, PA), anti-rabbit IgG, F(ab)₂ fragment (Jackson ImmunoResearch Laboratory), antimouse IgG1 (PharMingen), or antimouse IgG (Jackson ImmunoResearch Laboratory). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed three times with PBS. The slides were mounted using a water and alcohol-based mounting medium (Universal mount, Research Genetics).

Quantification of Intensity of Immunostaining. The intensity of the immunostaining of bFGF, VEGF, IL-8, and MMP-9 was quantified in three different areas of each sample by an image analyzer using Optimas image analysis software program (Media Cybernetics) to obtain an average measurement. Results are presented as a ratio between the expression by the tumor and by normal mucosa (which was set at 100; Ref. 7).

Quantification of MVD. MVD was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies according to the procedure of Weidner *et al.* (29). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The tissue was photographed using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goleta, CA) linked to a computer and digital printer (Sony Corporation). The MVD was expressed as the average number of the five highest areas identified within a single $\times 200$ field.

TUNEL Assay. For the TUNEL assay, tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with distilled water with Brij and treated with a 1:500 dilution of proteinase K (20 $\mu\text{g/ml}$) for 15 min; endogenous peroxidase was blocked

Table 2 mRNA and protein expression and MVD after therapy with monoclonal antibody C225 and/or paclitaxel

Therapy	mRNA expression index ^a				Protein expression index ^b				MVD ^c (per ×200 field)
	bFGF	VEGF	IL-8	MMP-9	bFGF	VEGF	IL-8	MMP-9	
Pretreatment CTRL	169	40	56	101	144	65	54	106	111 ± 22
CTRL (PBS)	100	100	100	100	100	100	100	100	104 ± 14
Paclitaxel	100	102	100	103	96	96	100	111	96 ± 17
C225	59 ^d	56 ^d	67 ^d	26 ^d	52 ^d	62 ^d	58 ^d	56 ^d	54 ± 14 ^d
Paclitaxel/C225	57 ^d	51 ^d	55 ^d	29 ^d	44 ^d	50 ^d	46 ^d	56 ^d	50 ± 14 ^d

^a The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of poly(dT) color reaction in each sample. The results are presented as the intensity of expression for each therapy compared with CTRL, defined as 100.

^b The intensity of the cytoplasmic immunostaining was quantitated by an image analyzer in three different areas of each sample to yield an average measurement and adjusted to the intensity of the cells of the tumors, with CTRL defined as 100.

^c MVD expressed as average number of five highest area identified within a single ×200 field.

^d $P < 0.005$ vs. CTRL (Mann-Whitney statistical comparison).

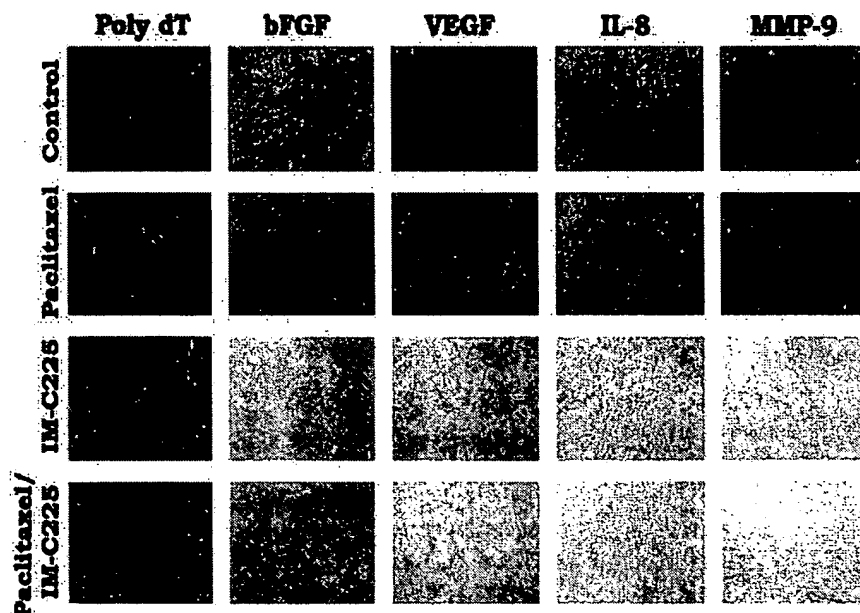


Fig. 4 The specific mRNA expression of bFGF, VEGF, IL-8 and MMP-9 was analyzed by ISH. Specific mRNA expression of angiogenic factors such as bFGF, VEGF, IL-8, and MMP-9 was reduced 40–70% in tumors treated with C225 (in both single-agent or combination therapy) compared with controls. Results represent one of two experiments. IM, ImClone.

using 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with distilled water with Brij and incubated for 10 min at room temperature with TdT buffer. Excess TdT buffer was drained, and the samples were incubated for 18 h at 4°C with terminal transferase and biotin-16-dUTP. The samples were then rinsed four times with Tris-buffer and incubated for 30 min at 37°C with a 1:400 dilution of peroxidase-conjugated streptavidin. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories), and washed three times with PBS. The slides were mounted using a Universal Mount (Research Genetics).

Quantification of Cell Proliferation and Apoptosis. Cell proliferation and apoptosis were determined by IHC of tissue sections with anti-PCNA antibodies and TUNEL assay. The tissue was photographed using a cooled CCD Optotronics

Tec 470 camera linked to a computer and digital printer. The density of proliferative cells and apoptotic cells was expressed as an average number of the five highest areas identified within a single ×200 field.

Immunofluorescence Double-Staining of Endothelial Cells and Apoptotic Cells. Frozen tissue sections (8 mm thick) were fixed with cold acetone for 5 min, acetone and chloroform (1:1, v/v) for 5 min, and acetone for 5 min. Samples were washed three times with PBS and incubated with protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min. The blocking solution was drained, and the samples were incubated with a 1:400 dilution of rat monoclonal anti-CD31 antibody (PharMingen; Ref. 29) for 24 h at 4°C. The samples were then rinsed with PBS three times for 3 min and incubated with protein-blocking solution for 10 min at room temperature. The blocking solution was again drained, and the samples were incubated for 1 h at

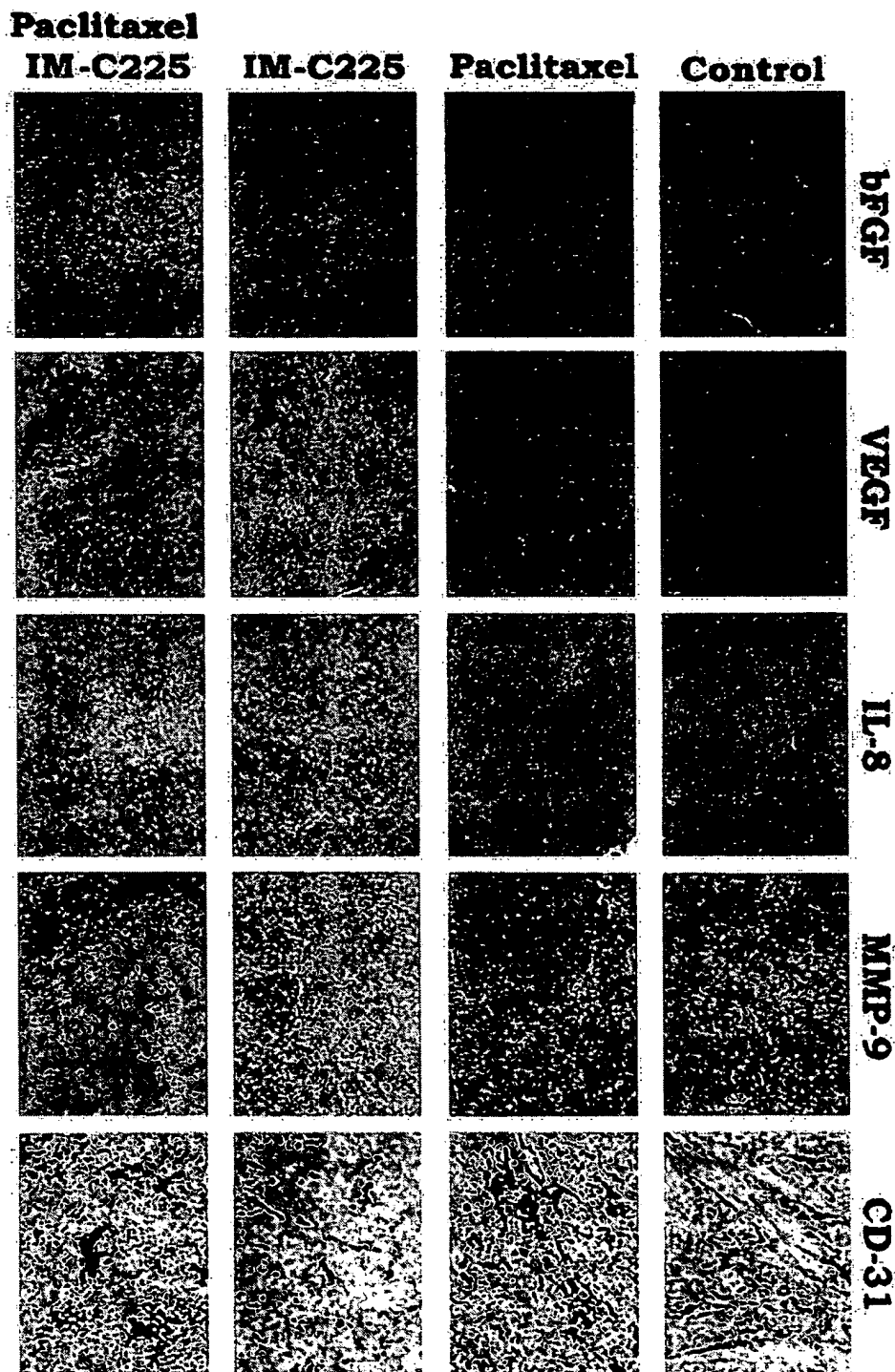


Fig. 5 The protein expression of bFGF, VEGF, IL-8, and MMP-9 was analyzed by IHC. Protein expression was 40–70% lower in tumors treated with C225 (in both single-agent and combination therapy) compared with controls. The number of CD31⁺ microvessels counted per $\times 200$ field in the bladder tumors was significantly lower in mice ($P < 0.005$) after therapy with C225 (54 ± 14) or paclitaxel/C225 (50 ± 14) compared with PBS (104 ± 14). Results represent one of two experiments. *IM*, ImClone.

room temperature with a 1:200 dilution of secondary goat antirat conjugated to Texas Red (Jackson ImmunoResearch Laboratory). The samples were then washed twice with PBS containing 0.1% Brij and then washed with PBS for 5 min. The TUNEL

assay was performed using a commercial kit (Promega, Madison, WI) with the following modifications. The samples were fixed with 4% paraformaldehyde for 10 min at room temperature. The samples were then washed with PBS twice for 5 min

Table 3 Apoptosis/PCNA ratio for cancer cells after therapy with monoclonal antibody C225 and/or paclitaxel

Therapy	Apoptosis index, ^a mean \pm SD (range) (per $\times 200$ field)	PCNA index, ^a mean \pm SD (range) (per $\times 200$ field)	Apoptosis/PCNA ratio, ^b (per $\times 200$ field)
Pretreatment CTRL ^c	2 \pm 1 (1–3)	303 \pm 98 (200–444)	0.7
CTRL (PBS)	4 \pm 1 (2–7)	248 \pm 68 (199–363)	1.6
Paclitaxel	14 \pm 4 ^d (11–18)	70 \pm 11 ^d (55–84)	20.0 ^d
C225	11 \pm 2 ^d (9–13)	87 \pm 16 ^d (69–109)	12.6 ^d
Paclitaxel/C225	25 \pm 6 ^{d,e} (19–33)	30 \pm 9 ^{d,e} (20–40)	83.3 ^d

^a The apoptosis index for cancer cells, determined by TUNEL assay, and cell proliferation, determined by IHC with PCNA, are expressed as the average of five highest areas identified within a single $\times 200$ field.

^b Apoptosis/PCNA ratio: mean percentage of the number of apoptotic cells divided by the number of PCNA-positive cells.

^c CTRL, control.

^{d,e} Mann-Whitney statistical comparison: ^d $P < 0.005$ vs. CTRL; ^e $P < 0.005$ vs. both single therapy groups with either paclitaxel or C225.

and incubated with 0.2% Triton X-100 for 15 min at room temperature. The samples were again washed with PBS twice (5 min each time) and incubated with equilibration buffer (from the kit) for 10 min at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mixture, and TdT enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h, avoiding exposure to light. The reaction was terminated by immersing the samples in 2 \times SSC for 15 min. The samples were then washed three times (5 min each time) to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 mg/ μ l Hoechst stain for 10 min at room temperature. The samples were then washed with PBS twice (5 min each time). To preserve fluorescence and reduce bleaching, Prolong solution (Molecular Probes, Inc., Eugene, OR) was used to mount coverslips. The slides were examined using an inverted microscope (model IX70; Olympus, Melville, NY). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green fluorescence within the nuclei of apoptotic cells. Results were expressed as the ratio of apoptotic endothelial cells to total endothelial cells in 10 random fields at $\times 40$ magnification.

Statistical Analysis. The statistical differences for the number of vessels, proliferative cells, and apoptotic cells and for staining intensity for EGFR and activated EGFR, bFGF, VEGF, IL-8, and MMP-9 within the bladder tumors were analyzed by the Mann-Whitney test. Incidences of tumor and metastasis were statistically analyzed by the χ^2 test. $P < 0.05$ was considered significant.

RESULTS

In Vitro Cell Growth Inhibition by C225 and Paclitaxel.

In vitro therapy with either C225 or paclitaxel for 72 h resulted in a dose-dependent antiproliferative effect as measured by the [³H]thymidine incorporation assay. The IC₅₀s of C225 and paclitaxel were 100 and 10 μ g/ml, respectively (Fig. 2A). At low concentrations, C225 (1.0 and 10 μ g/ml) enhanced the antiproliferative effects of paclitaxel (Fig. 2B).

In Vitro Induction of Apoptosis by C225 and Paclitaxel.

The results were expressed as the ratio of apoptotic to total cells. Treatment with paclitaxel alone induced apoptosis in 29.9, 50.3, and 54.4% of the 253J B-V cells at concentrations of 1, 10, and

100 μ g/ml, respectively. C225 did not significantly enhance *in vitro* apoptosis (Fig. 3).

Inhibition of Growth and Metastasis of Established Human TCC. In the control mice injected with the 253J B-V cells and killed on day 21 (the day treatment began), tumors had a median weight of 269 mg (range, 144–378 mg). Treated mice were closely monitored for any signs of progressive disease and killed if they became moribund. The effects of treatment on tumor growth and metastasis are summarized in Table 1. Treatment with paclitaxel followed 2 days later by C225 (median bladder weight, 85 mg; range, 69–133 mg), was significantly more effective at eradicating bladder tumors than was treatment with C225 alone (median bladder weight, 168 mg; range, 72–288 mg; $P < 0.05$), paclitaxel alone (median bladder weight, 273 mg; range, 83–563 mg; $P < 0.005$) or PBS alone (median bladder weight, 481 mg; range, 377–877 mg; $P < 0.0005$). Combination therapy also significantly reduced spontaneous lymph node metastasis (no animal had metastases) compared with treatment using paclitaxel alone (seven of nine animals; $P < 0.005$) or PBS alone (nine of nine animals; $P < 0.0001$). Although two of seven mice treated with C225 alone developed spontaneous lymph node metastases, this result did not significantly differ from results following combination therapy.

Inhibition of bFGF, VEGF, IL-8 and MMP-9 Expression and MVD after EGFR Blockade and Paclitaxel. Expressions of bFGF, VEGF, IL-8, and MMP-9 mRNA and protein were analyzed by ISH (Table 2 and Fig. 4) and IHC (Table 2 and Fig. 5), respectively. The mRNA and protein expression of bFGF, VEGF, IL-8, and MMP-9 was 40–70% lower in tumors of mice treated with C225 alone or with C225 in combination with paclitaxel, compared with PBS-treated controls ($P < 0.005$; Table 2 and Fig. 4).

MVD was significantly lower in tumors of mice treated with C225 as a single agent or in combination with paclitaxel than in tumors from PBS-treated controls (54 \pm 14 for C225 alone, 50 \pm 14 for paclitaxel/C225 combination therapy compared with 104 \pm 14 for untreated controls; $P < 0.005$). Paclitaxel alone had no effect on MVD and did not enhance the reduction in MVD seen after C225 therapy alone (Fig. 5).

Enhancement of Apoptosis and Inhibition of Proliferation by Therapy with C225 and Paclitaxel. We evaluated the effect of therapy with C225 and paclitaxel on cellular proliferation and apoptosis by IHC for PCNA and TUNEL,

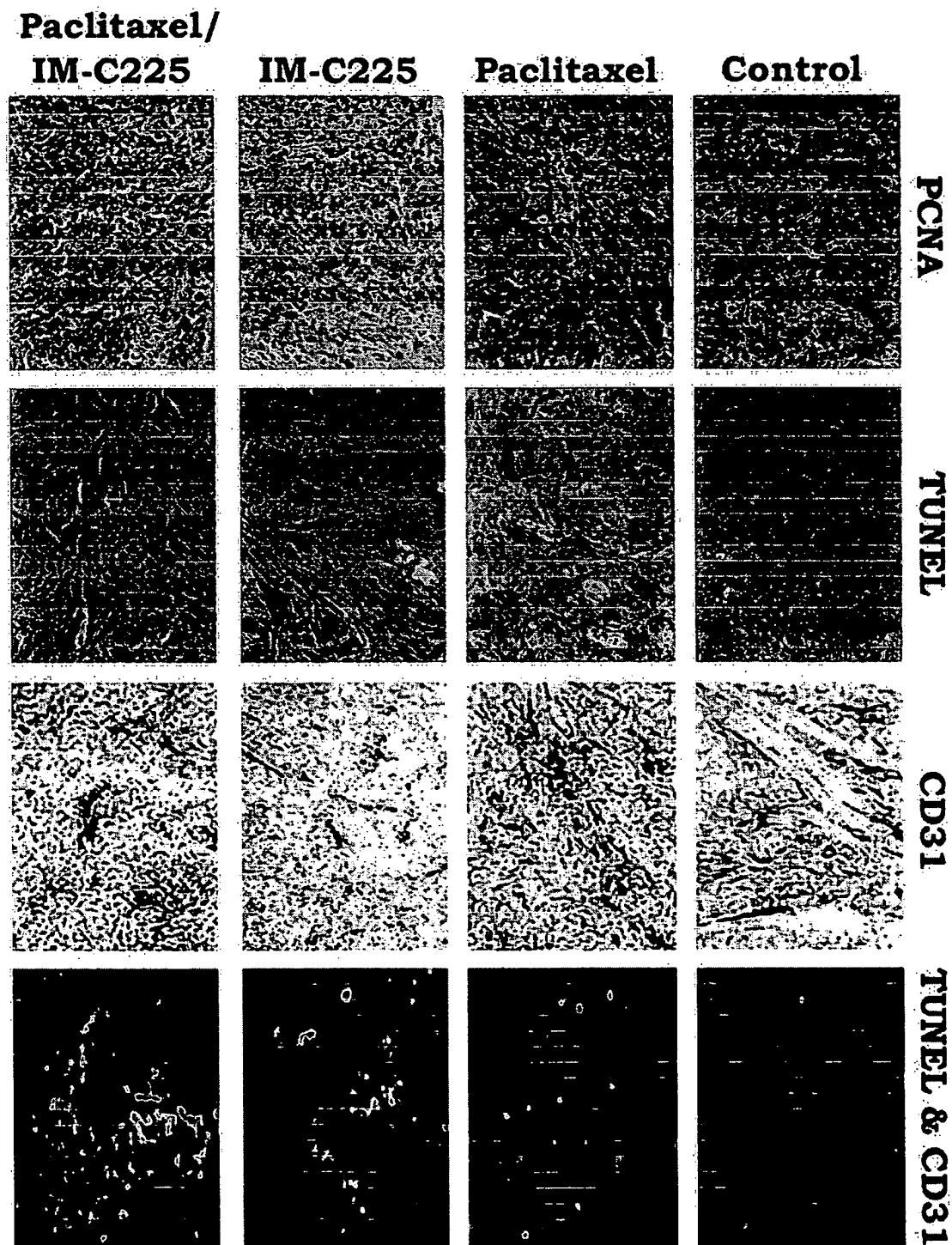


Fig. 6 The expression of apoptosis in cancer cells and endothelial cells of microvessels was analyzed by IHC with anti-PCNA antibody, TUNEL assay, and immunofluorescence double-staining of endothelial cells and apoptotic cells. Results represent one of two experiments. *IM*, ImClone.

Table 4 Apoptosis of endothelial cells of microvessels after therapy with monoclonal antibody C225 and/or paclitaxel

Therapy	Apoptosis index of endothelial cells ^a Mean \pm SD (Range) (% per $\times 400$ field)
Pretreatment CTRL	0.4 \pm 0.9 (0.0–2.0)
CTRL (PBS)	0.7 \pm 1.5 (0.0–3.3)
Paclitaxel	11.8 \pm 3.8 (8.8–16.3) ^c
C225	24.0 \pm 9.1 (14.3–35.3) ^c
Paclitaxel/C225	49.5 \pm 11.3 (37.5–63.2) ^{c,d}

^a The apoptosis of endothelial cells of microvessels, as determined by immunofluorescence double-staining, is expressed as the average percentage of apoptosis in all endothelial cells of the five highest MVD areas identified within a single $\times 200$ field.

^b CTRL, control.

^{c,d} Mann-Whitney statistical comparison: ^c $P < 0.005$ vs. CTRL; ^d $P < 0.005$ vs. any other groups.

respectively. The number of PCNA-positive cancer cells per $\times 200$ field was significantly lower in tumors from mice treated with paclitaxel alone (70 ± 11) or with C225 alone (87 ± 16) than in tumors from PBS-treated controls (248 ± 68 ; $P < 0.005$). The combination of C225 and paclitaxel significantly inhibited proliferation compared with the use of each agent alone, with the greatest difference seen after initial therapy with paclitaxel, followed by C225 (30 ± 9 ; $P < 0.005$).

The number of apoptotic cancer cells per $\times 200$ field significantly increased, from 4 ± 1 in PBS-treated controls to 14 ± 4 and 11 ± 2 following therapy with C225 and paclitaxel, respectively ($P < 0.005$; Table 3 and Fig. 6).

Induction of Endothelial Cell Apoptosis after Therapy with C225 and Paclitaxel. We evaluated whether endothelial cells underwent apoptosis after therapy with C225 by dual immunofluorescence with anti-CD31 and TUNEL antibodies (Fig. 6). Endothelial cells were detected by localized red fluorescence, whereas green fluorescence was detected within the nuclei of apoptotic cells. Double-labeling of endothelial cells undergoing apoptosis resulted in localized yellow fluorescence. The number of apoptotic endothelial cells counted per $\times 400$ field was calculated as the ratio of double-labeled to total endothelial cells. We observed significantly higher apoptotic indices for endothelial cells after treatment with either paclitaxel or C225 ($P < 0.005$). Treatment with paclitaxel and C225 resulted in an increase in the apoptotic index to 49.5 ± 11.3 , which was significantly higher than values for all other therapy groups ($P < 0.005$; Table 4). Clusters of apoptotic tumor cells were seen surrounding apoptotic endothelial cells when the two agents were combined (Fig. 6).

DISCUSSION

Although TCC of the bladder is a chemosensitive tumor, most deaths from bladder cancer are caused by metastases that are resistant to conventional chemotherapy (30, 31). Although up to 70% of patients with advanced TCC have an initial response to chemotherapy, in most of these, chemoresistant disease will develop rapidly (32). Therefore, the development of novel chemotherapeutic strategies is necessary if the outcome for patients with advanced bladder cancer is to improve.

Recent reports indicate that biological agents such as an-

tagonists of EGFR or HER-2/neu receptor modulate host responses and enhance the efficacy of standard cytoreductive chemotherapy (7, 33). The anti-EGFR antibody C225 has significant antitumor activity against a number of human malignancies, including those of the prostate (34), breast (8), colon (8), ovary (8), and kidney (35), and TCC of the bladder (7). This antitumor effect is modulated by cell cycle regulation via induction of apoptosis and by inhibition of invasion and tumor-induced angiogenesis (36–39). Both C225 and the anti-HER2 monoclonal antibody 4D5 have been combined with doxorubicin and paclitaxel to effectively treat breast and ovarian cancers (8, 9). Treatment of human breast xenografts with the HER-2/neu tyrosine kinase inhibitor Emodin sensitized these tumors to paclitaxel and improved the survival of mice treated by this combination. C225 has also been combined with the monoclonal antibody 4D5 to treat ovarian cancer (33).

In the present study, we evaluated the efficacy of using systemic C225 and paclitaxel to treat human TCC growing within the bladder of nude mice. Paclitaxel demonstrates a significant antitumor effect against TCC of the bladder, both as a single agent and in combination with other cytotoxic agents. Paclitaxel increases microtubule stability by preventing tubulin depolymerization, which results in tubulin bundling (13, 14). These cytoskeleton changes lead to cell cycle arrest and apoptosis within 20 h of paclitaxel exposure (40, 41). The extent of paclitaxel-induced apoptosis is regulated by Raf-1 activity (42, 43), such that 2–4-fold increases in paclitaxel-induced cytotoxicity occur if Raf-1 kinase levels are reduced. Therefore, we treated human TCC growing within the bladders of athymic nude mice with systemic paclitaxel in combination with C225, an agent that down-regulates Raf-1 activity.

In vitro, C225 enhanced the antiproliferative and apoptotic effects of paclitaxel in a dose-dependent manner. The antiproliferative effect of C225 was 25% at a dose of 1 $\mu\text{g}/\text{ml}$, similar to that of paclitaxel at a dose of 0.1 $\mu\text{g}/\text{ml}$. When the drugs were combined at that dose, there was an additive effect on cytostasis that approached 55%. Similarly, we observed an additive effect on the induction of apoptosis. Combined use of C225 and paclitaxel at their IC_{50} doses (100 $\mu\text{g}/\text{ml}$ for C225 and 10 $\mu\text{g}/\text{ml}$ for paclitaxel) resulted in apoptosis in 64.5% of the tumor cells compared with 50.3% for paclitaxel alone at 10 $\mu\text{g}/\text{ml}$ and 8.5% for C225 alone at 100 $\mu\text{g}/\text{ml}$.

The benefit of combining C225 with paclitaxel became more apparent after *in vivo* treatment. In a previous study (7), we showed that interruption of EGFR signaling pathways with C225 caused tumor regression and inhibited metastasis of established human TCC growing within the bladders of athymic nude mice. This significant antitumor effect was mediated by the induction of apoptosis and the inhibition of angiogenesis. In the present study, we observed that the cytotoxic and proapoptotic effects of C225 were enhanced by the administration of paclitaxel. This therapy was also very effective in preventing metastasis. Although we could not demonstrate a statistically significant difference compared with treatment using C225 alone, no mice that received initial treatments with paclitaxel followed by C225 developed spontaneous metastasis, whereas two mice treated with C225 alone developed lymph node metastases.

The mechanisms responsible for the interaction of C225

and paclitaxel are unclear. Tumor-induced neovascularization and invasion were inhibited by treatment with C225, secondary to down-regulation of VEGF, bFGF, IL-8, and MMP-9 expression by the 253J B-V tumor cells. Although the down-regulation of invasion and metastasis is an essential function of this therapy, it was not enhanced by the addition of paclitaxel.

It has been hypothesized that therapy with paclitaxel might functionally up-regulate EGFR or the HER2/neu receptor and render cells more susceptible to C225 (44, 45). However, although our data indicated that neither EGFR nor activated EGFR is up-regulated by the use of paclitaxel alone, activated EGFR was down-regulated when paclitaxel was administered prior to C225. It is possible that we failed to recognize the functional up-regulation of EGFR because of the time at which we made our measurements (data not shown).

The most likely mechanism responsible for the increased antitumor activity of the combined treatment with C225 and paclitaxel is an increase in apoptosis coupled with a decrease in proliferation. This is the result of the sum of effects on separate pathways regulating apoptosis (15, 16, 40, 41, 46). Apoptosis was markedly increased when paclitaxel was administered before C225. We hypothesize that C225-mediated cell cycle arrest limits paclitaxel-induced apoptosis. The ratio of apoptosis to PCNA appears to be a marker that predicts response to this therapy. The ratios in the tumors treated initially with paclitaxel were 4.2-, 6.6-, and 52.1-fold higher than the ratios for paclitaxel or C225 alone and PBS-treated controls, respectively, and 2.4-fold higher than the ratios for initial treatment with C225. Using double-labeling fluorescence, we found that therapy with C225 and paclitaxel induced apoptosis to a lesser degree in endothelial cells as well as tumor cells. This effect became more significant in the group of mice treated initially with paclitaxel, in which we observed clusters of apoptotic tumor cells surrounding apoptotic endothelial cells.

In summary, our experiments demonstrated that paclitaxel enhances the antitumor effect of C225 on human bladder TCC growing within the bladders of athymic nude mice and demonstrated the benefit of combining two therapeutic modalities that have entirely different mechanisms of action. The improved response to this strategy seems to result from enhancement in the induction of apoptosis by paclitaxel and C225.

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